Phytopathol. Mediterr. (2009) 48, 439–446

The effect *in vitro* of exogenously applied *p*-hydroxybenzoic acid on *Fusarium oxysporum* f. sp. *niveum*

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Summary. The allelochemical, *p*-hydroxybenzoic acid, was applied *in vitro* to *Fusarium oxysporum* f. sp. *niveum*, a pathogen causing watermelon wilt disease, by adding the acid to the medium. *P*-hydroxybenzoic acid slightly stimulated the growth of the fungus at low concentrations (200–800 mg L^{-1}), but it strongly inhibited its growth at the highest concentration (1600 mg L^{-1}). At this acid concentration the mycelial mass was reduced by 63.7% and the conidial germination rate was decreased by 46.9–100%. Sporulation was also suppressed, with a 91.4% reduction. However, fusaric acid production by *Fusarium oxysporum* f. sp. *niveum* was stimulated with an increase of 47.3–379.4%. The activity of proteinase, pectinase and cellulase was stimulated by *p*-hydroxybenzoic acid, but amylase activity was depressed.

Key words: biomass; Fusarium wilt of watermelon; virulence factors.

Introduction

Watermelon production with a long-term monoculture system has led to the frequent occurrence of a wilt of watermelon caused by *Fusarium oxysporum* f. sp. *niveum* which is very difficult to remove from the soil once it has been introduced. The fungus and its wilt severely impact watermelon production. It is the most important soilborne pathogen limiting watermelon production in many areas of the world (Martyn, 1996; Hao *et al*., 2006; Wu *et al*., 2008a,b).

Fusarium species cause vascular diseases in a number of plants, such as watermelon, cucumber, tomato, pepper, muskmelon, bean and cotton (McKeen *et al*., 1961; Armstrong and Armstrong, 1981; Nelson, 1981; Gordon *et al*., 1997). More than 50% of isolates of known *Fusarium* species are toxigenic and produce harmful secondary metabolites (Marasas *et al*., 1984). *Fusarium* species damage host plants through the penetration of the hyphae into the host vascular tissues, secretion of hydrolytic enzymes related to pathogenesis, mycotoxin production and the cellular apoptosis of the host cells as the infection progresses (Gaumann, 1957; Fuchs *et al*., 1965; Booth, 1971; Abraham, 1986; Bacon *et al*., 1996; Abbas *et al*., 1997; Pavlovkin *et al*., 2004).

Much attention has been paid to the effect that *F: oxysporum* has on host plants. but much less to the effect of host plants on the pathogen as they interact with it. However, pathogen invasion tends to produce root exudates and decaying plant residues. A substantial number of plant secondary metabolites are directly or indirectly involved in protecting the plants against insect herbivores and pathogens (Moraes *et al*., 2008). Many kinds of root exudates and decaying residues (decomposing litter) are

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phytotoxic due to allelochemicals released from the root exudates and decaying residues. These allelochemicals have been isolated and identified as organic acids, especially phenolic acids, such as cinnamic, vanillic, coumaric, and benzoic acid (Ohno *et al*., 2001; Yu *et al*., 2003; Asao *et al*., 2003; Hao *et al*., 2006; Lee *et al*., 2006; Wu *et al*., 2008a,b). Phenolic acids such as hydroxycinnamic acids ester-linked to polysaccharides frequently occur in plants, and large amounts of simple phenolic acids, such as benzoic, *p*-coumaric and syringic acids, are released during the aerobic catabolism of lignin (Blackley and Simpson, 1964; Toms and Wood, 1970; Kuwahara, 1980). Root exudates and decaying plant residues variously affect methanogenic microbial communities on rice or maize roots and in the rhizosphere (Baudoin *et al*., 2003; Lu *et al*., 2004), the composition of bacterial communities (Landi *et al*., 2006; Prosser *et al*., 2006; Sturz, 2006), and the microbial biomass (Sung *et al*., 2006). Ginseng root exudates stimulate the growth of *Phytophthora cactorum* and *Pythium irregulare* (Nicol *et al*., 2003), rhizobacterial populations (Nehl *et al*., 1997; Sturz and Christie, 2003; Prosser *et al*., 2006) and fungal species that are abundant in the desert (Mandeel, 2002).

Some authors suggest that root exudates initiate and manipulate biological and physical interactions between the roots and soil-borne organisms, and thus play an active role in root-microbe communication (Bais *et al*., 2004). However, the effect of specific compounds from root exudates or decaying plant residues on specific pathogens is scarcely known, though experimental studies have applied various chemicals, such as benzoic, caffeic and vanillic acids to the soil to determine the effects of phenolic acids on the microbial biomass and populations (Udo and Shafer, 1988; Udo, 1997).

p-hydrioxybenzoic acid is one of the phytotoxic allelochemicals contained in plant root exudates, and is one of the products of the reaction of L-phenylalanine ammonia-lyase with secondary metabolites inside the plant (Bolwell *et al*., 1986; Wu *et al*., 2001; Yu *et al*., 2003). It accumulates in cytosol and in the cell walls of the plant (Sircar *et al*., 2007; Chen *et al*., 2008; Sircar and Mitra, 2008). It is one of the components bound to the plant cell wall and is a precursor in lignin formation, and has been suggested to be involved in cell wall extensibility, which plays a key role in cell defense (Bokern, 1991; Locher *et al*., 1994; Wallace and Fry, 1999; Yu *et al*., 2006;). Some experiments have shown that *Bacillus subtilis, B. cereus* and *Staphylococcus aureus* are inhibited by cumin extract (Ani *et al*., 2006). The phenolic acid decarboxylase gene of the *Bacillus* sp. BP-7 has been cloned (Prim *et al*., 2003). The mycelial growth of *F. oxysporum* f. sp. *albedinis* is inhibited by cell wall-bound phenolics in resistant cultivars of date palm roots (Elmondafar and Boustani, 2001). Colony growth rates of *Mollisia* sp., *Penicillium commune*, *Mortierella* sp., *Trichoderma koningii*, *Trichoderma* sp. and *Phoma herbarum* have also been tested in soil containing *p*-hydroxybenzoic acid (Hughes *et al*., 2007).

p-hydroxybenzoic acid can be utilized by *Azotobacter chroococum* and *Lactobacillus plantarum* as a carbon source (Juarez *et al*., 2005; Landete *et al*., 2008; Rodriguez *et al*., 2008). However, to our knowledge, little is known about the direct effect of *p*-hydroxybenzoic acid on *F. o.* f. sp. *niveum*. There may exist a link between *p*-hydroxybenzoic acid and the invasion of plants by the fungus.

Our goal was to test the allelopathic potential of *p*-hydroxyybenzoic acid applied to *F. o.* f. sp. *niveum,* in terms of the host-specific pathogen and the magnitude of the allelopathic interactions, and to investigate *in vitro* whether there is a relationship between *p*-hydroxybenzoic acid and the pathogenic factors of *F. o.* f. sp. *niveum*, where fusarium wilt of watermelon can be controlled using this acid together with the genetic modification of watermelon obtained in breeding programmes.

Materials and methods

Fungal strain and chemicals

Fusarium o. f. sp. *niveum* was isolated from infected watermelon roots grown in a greenhouse plot, in accordance with Koch's postulates, by the Laboratory of Plant-Microbe Interactions, Nanjing Agricultural University, China.

p-hydroxybenzoic acid and the other main chemicals (analytical grade) used in the experiment were obtained from the Sigma Co. (St. Louis, MO, USA).

Measurement of *F. o.* f. sp. *niveum* colony growth

A 5-mm agar plug taken from a 7-day-old potato dextrose agar (PDA, Fujian agar co., China) culture was inoculated in the center of the plate and was incubated at 28°C for 7 d. Colony diameter was measured three times on each plate after incubating for 3 and 7 d.

Assessment of conidial germination

To determine the effect of *p*-hydroxybenzoic acid on conidial germination, *F. o.* f. sp. *niveum* was grown on 2% water agar. A 5-mm agar plug taken from a 7-day-old PDA culture was inoculated in a potato dextrose liquid culture and incubated at 28°C for a further 7 d. The broth was filtered to remove the conidia. The conidial suspension was diluted to ≤200 conidia per milliliter with sterile distilled water. A 0.1 ml diluted suspension was spread on plates

and incubated at 28°C for 3 d. The number of colonies was counted daily.

Determination of sporulation

Sporulation was determined by growing *F. o.* f. sp. *niveum* (as described above) in Bilay and Joffe's medium (Booth, 1971) with minor modifications (using 4.0 g CMC-Na instead of 15 g CMC, and adjusting the pH to 4 with 2 mol L^{-1} HCl). After incubating for 7 d, 0.1 ml culture broth, diluted to 10^{-5}– 10^{-7}, was spread on PDA. Dishes were incubated at 28°C in the dark for 4 d, after which the colonies were counted and converted to the number of conidia in a liquid culture.

Measurement of mycelial mass and enzyme activity

Fusarium o. f. sp. *niveum* was grown in 100 ml flasks filled with 30 ml potato dextrose broth adjusted to pH 4.5 with 2 mol L^{-1} HCl, and was inoculated with a 5-mm agar plug from a 7-day-old PDA culture. Cultures were incubated in a shaker (170 rpm) at 28°C for 7 d. The fungal biomass (dry weight) was determined after filtration and drying at 80°C for 12 h, when constant weight was achieved.

Protease activity was assayed as described by Tseng and Mount (1974). One unit of protease activity was defined as a 0.001 increase in absorbance per minute under the assay conditions. Pectinase activity (mainly that of polygalacturonase) was assayed by the DNS method (Silva *et al*., 2005). One unit of pectinase was defined as the amount of β-galacturonic acid hydrolyzed from pectin per minute under the assay conditions. Cellulase activity was also determined by the DNS method (Berlin *et al*., 2005). One unit of cellulase activity was defined as the amount of cellulase that produced 1 µmol of reduced sugar per minute under the assay conditions. Amylase activity was also assayed by the DNS method (Murado *et al*., 1997). One unit of amylase activity was defined as the amount of amylase that released 1 mg of reducing sugars (glucose equivalents) per minute under the assay conditions.

Extraction and determination of fusaric acid

Fusaric acid production (mainly fusaric acid) was determined from the growth in Richard's medium (Gaumann, 1957), as described above but with a 12 h photoperiod under fluorescent light for 35 d. Broth was acidified to pH 2 with 2 mol L^{-1} HCl, mixed with an equal volume of ethyl acetate, vigorously shaken for 2 min, allowed to settle for 30 min, and then the organic phase was removed. After repeating this procedure 5 times, the organic phase was centrifuged for 10 min at 5000 *g*. The supernatant was dried and condensed at ≤40°C in a spinning evaporator. The dried residue was re-dissolved in 5 ml of ethyl acetate and the OD_{268} (Matsui and Smith, 1988) was determined by a UV-120-02 spectrophotometer (Shimadzu, Japan).

Experimental design and statistical analysis of data

Based on our preliminary experiments, studies were carried out using five concentrations of *p*-hydroxybenzoic acid: 0, 200, 400, 800 and 1600 mg. L^{-1}. The control used 2 ml of sterilized ethyl acetate and no *p*-hydroxybenzoic acid. The *p*-hydroxybenzoic acid solution was filter-sterilized by a 0.22 µm pore membrane (Millipore). Data were analyzed with Microsoft Excel™. The values were represented as the mean of three replicates (mean ± SD) for each treatment. ANOVA was used to detect significant differences between treatments, and multiple comparison was carried out with SPSS version 11.5 (Chicago, IL, USA). Significant differences were found between treatments, and between treatments and controls.

Results

Effect of p-hydroxybenzoic acid on the mycelial mass and on colony growth

The mycelial mass of *F. o.* f. sp. *niveum* in a potato dextrose liquid culture and the colony growth of the fungus on PDA were inhibited by *p*-hydroxybenzoic acid. At lower concentrations of *p*-hydroxybenzoic acid (200–800 mg L^{-1}), the growth of the fungus was reduced by 15.9–28.1% dry weight compared with the untreated control, while at the highest concentration (1600 mg L^{-1}) the dry weight of the fungus was decreased by 63.7% (n=3, F=1.586, *P*=0.576) (Figure 1A). Colony growth was strongly suppressed, with a reduction of 6.9–34.7% compared with the control (n=3, F=1.989, *P*=0.814) (Figure 1B).

Effect of p-hydroxybenzoic acid on conidium germination and sporulation

Conidium germination on Petri dishes and conidium formation in liquid culture was severely inhibited by *p*-hydrobenzoic acid even at a very low concentrations. Conidium germination was reduced by 46.9–100% (n=3, F=6.388, *P*=0.736) (Figure 1C). The number of conidia produced in a shaker declined by up to 91.4% (n=3, F=8.572, *P*=0.473) (Figure 1D).

Effect of *p*-hydroxybenzoic acid on fusaric acid production

Fusaric acid production of *F. o.* f. sp. *niveum* in a liquid culture was greatly stimulated by *p*-hydroxybenzoic acid depending on the concentration of the acid. At acid concentrations ranging from 0–1600 mg L^{-1}, the yield of fusaric

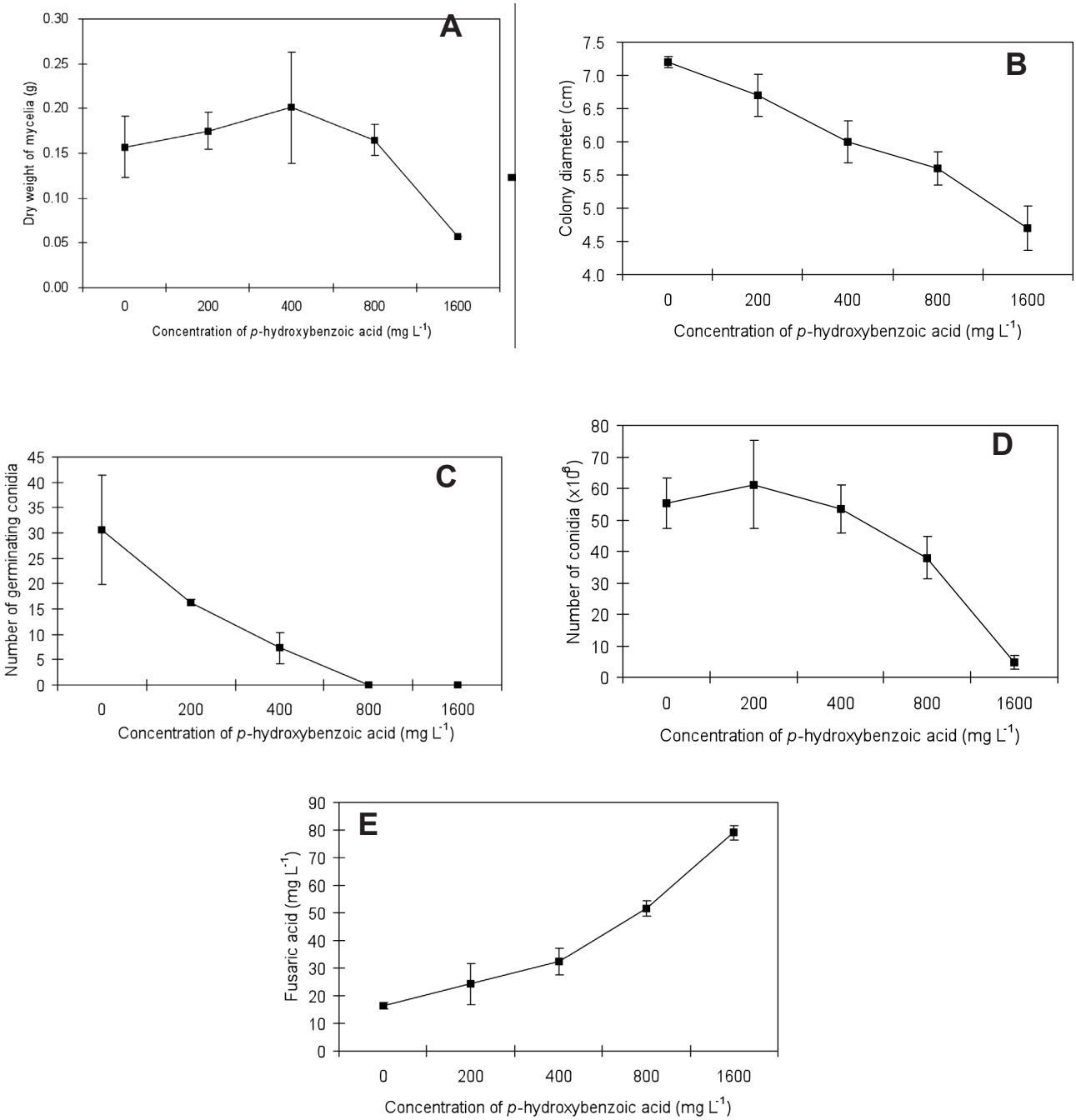

Figure 1. Effect of *p*-hydroxybenzoic acid on *Fusarium oxysporum* f. sp. *niveum*. Concentrations of 0, 200, 400, 800 and 1600 mg L^{-1} of *p*-hydroxybenzoic acid were tested. Bars indicate standard deviation of three replicates for each treatment. A) Effect on mycelial mass. The inoculated fungus was incubated at 28°C, 180 rpm. B) Effect on colony growth. The colony was grown on PDA at 28°C. C) Effect on conidia germination. The conidia were germinated on PDA without dextrose at 28°C. D) Effect on sporulation of *F. o.* f. sp. *niveum* in a potato dextrose liquid culture at 28°C, 180 rpm. E) Effect on fusaric acid production. The inoculated fungus was incubated at 28°C, 180 rpm.

acid increased by 47.3–379.4% (n=3, F=3.759, *P*=0.426) (Figure 1E).

Effect of p-hydroxybenzoic acid on the activity of enzymes related to pathogenesis

The activity of proteinase, pectinase and cellulase was boosted by *p*-hydroxybenzoic acid in a liquid culture, whereas amylase activity was depressed. Generally, the activity of enzymes first went up, and peaked, and then went down again. Proteinase activity increased by 42.7–64.7% compared with the untreated control (Figure 2a). At lower concentrations of *p*-hydroxybenzoic acid (200–400 mg L-1), pectinase activity was little changed, but an increase of 219.2% in activity occurred at higher concentrations (Figure 2b). *P*-hydroxybenzoic acid strongly stimulated cellulase activity, with an increase of 2.3–745.7% at different concentrations (Figure 2c). Amylase activity decreased only slightly (Figure 2d).

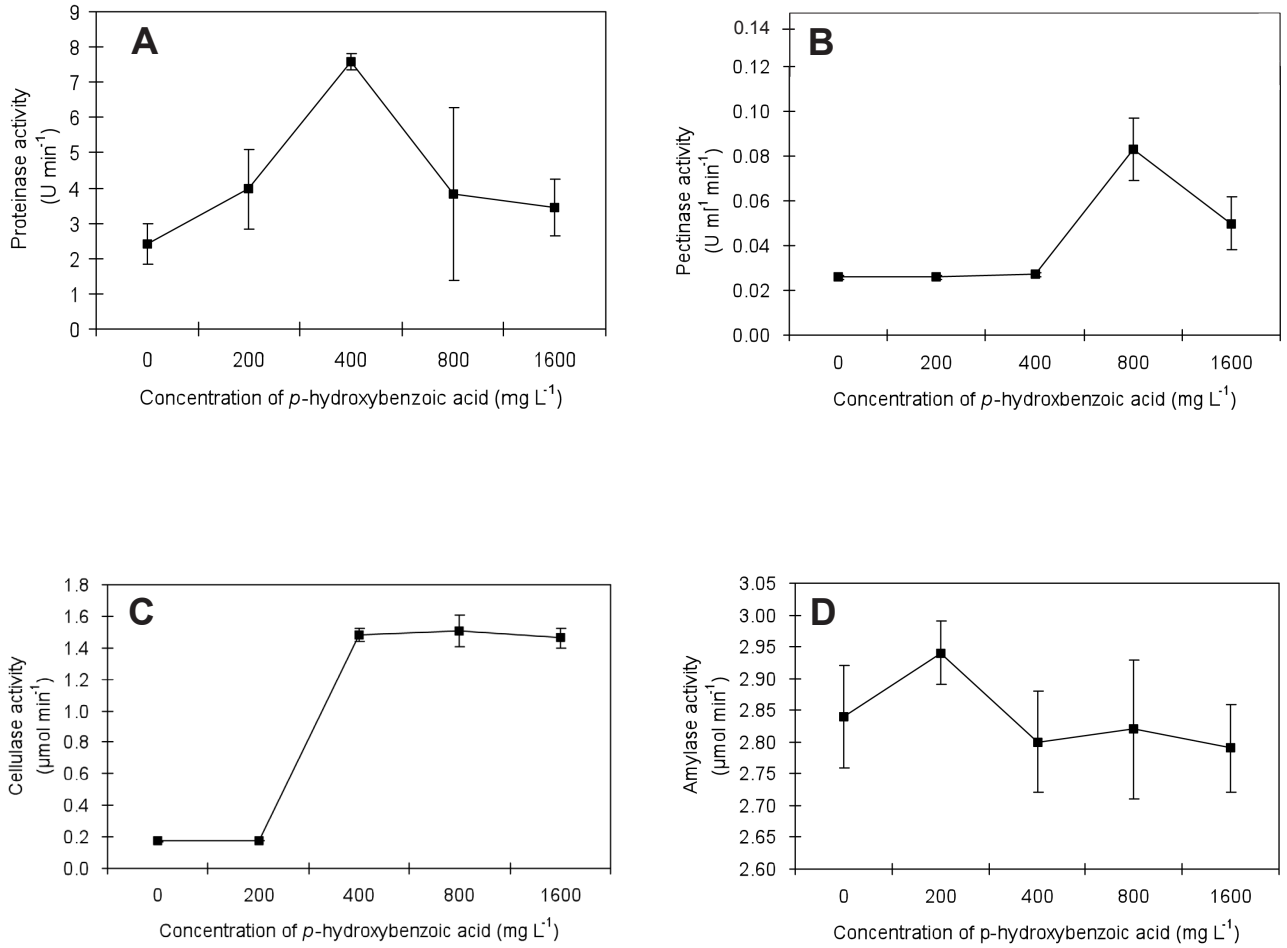

Figure 2. Effect of *p*-hydroxybenzoic acid on phytopathogenic enzyme activity of *Fusarium oxysporum* f. *sp. niveum* in a potato dextrose liquid culture at 28°C, 180 rpm: A, proteinase; B, pectinase; C, cellulase; D, amylase. 0, 200, 400, 800 and 1600 mg L^{-1} of *p*-hydroxybenzoic acid were used. Bars indicate standard deviation of three replicates for each treatment.

Discussion

P-hydroxybenzoic acid is often found in root exudates and decaying plant tissues, and this may serve as a signal transduction molecule in the plant-microbe interaction (Ohno *et al*., 2001; Asao *et al*., 2003; Yu *et al*., 2003; Lee *et al*., 2006; Hao *et al*., 2006). It strongly inhibited growth but stimulated the virulence factors of *F. o.* f. sp. *niveum*. In the present study, the growth of *F. o.* f. sp. *niveum* was inhibited by *p*-hydroxybenzoic acid, depending on the acid concentration, with the biomass in liquid culture decreasing by 63.7% (Figure 1). This was consistent with the finding that the mycelial growth of *F. o.* f. sp. *albedinis* was inhibited by cell wall-bound phenolics in resistant cultivars of date palm roots (Elmondafar and Boustani, 2001), and with the finding that *p*-hydroxybenzoic acid inhibited the hyphal extension of fungi in Antarctic soil (though growth rates increased) (Hughes *et al*., 2007), and also with the fact that the growth of *Lactobacillus plantarum* and *Azotobacter chroococcum* was stimulated by *p*-hydroxybenzoic acid (Juarez *et al.*, 2005; Landete *et al.*, 2008; Rodriguez *et al.*, 2008). Conidia germination was strongly suppressed even at the lower acid concentrations, with no germinating conidia seen at concentrations from 800–1600 mg L^{-1} (Figure 3).

In this investigation, sporulation by *F. o*.f. sp. *niveum* in a liquid culture was also strongly suppressed. This is not consistent with the report that fungal populations are stimulated by phenolic acids added to the soil (Udo and Shafer, 1988). The results on sporulation were confirmed by other studies which reported that cinnamic, vannilic and coumaric acid inhibited *F. o.* f. sp. *niveum* but repressed spore formation of this fungus (Wu *et al.*, 2008a,b; Wu *et al.*, 2008).

In contrast to fungal growth, which went down, conidium formation and germination, and fusaric acid production by *F. o.* f. sp. *niveum* were all stimulated (Figure 5). It might seem contradictory that suppressed growth should be accompanied by increased conidium formation and germination in *F. o*. f. sp. *niveum*. However, the physiological mechanism causing poor growth may differ from those mechanisms boosting fusaric acid production, and the conidium formation and germination of *F. o.* f. sp. *niveum* under biotic and abiotic stress. This would be consistent with the reported finding that cinnamic, vanilic and coumaric acids inhibited the growth of *F. o.* f. sp. *niveum* but stimulated fusaric acid production (Wu *et al*., 2008a,b; Wu *et al*., 2008).

Fusaric acid is a strong agent that is produced when *Fusarium* sp. invades watermelon or other plants, and it is an important virulence factor causing plant wilting. As is well known, mycotoxins are secondary metabolites produced by a number of fungal species while phytotoxins are metabolites produced by plants, such as watermelon, cucumber, tomato, beans and cotton (Gaumann, 1957). Fusaric acid is a wilt toxin on tomato plants infected with *F. o.* f. sp. *lycopersici,* and the toxic concentration that causes wilting is 150 mg L^{-1} (David, 1969). The toxins produced by pathogens are primary determinants of pathogenesis, when they act as the key element initiating infection and symptoms. They are secondary determinants when they only modify the symptoms, even if they do so very strongly (Lepoivre, 2003). Moderate doses of fusaric acid (a fusarial mycotoxin) cause apoptosis in saffron, while high doses of this acid produce necrosis (Leili and Behboodi, 2006).

Other virulence factors equally important for *Fusarium* spp. are the enzymes related to pathogenesis. Pectinases and cellulases released by phytopathogenic fungi stimulate the infection process in many plant diseases. They facilitate the penetration of the fungus into the plant by the hydrolytic cleavage of polymers (pectic substances, cellulose), which constitute the plant cell walls (Fuchs *et al*., 1965)**.** Plants are damaged when Fusarium hyphae penetrate the host vascular tissues and when the fungus secretes hydrolytic enzymes related to pathogenesis, and produces fusaric acid during infection (Fuchs *et al*., 1965; Booth, 1971; Joffe, 1986). In the present study, proteinase, pectinase and cellulase activity was stimulated by *p*-hydroxybenzoic acid with increases of 42.7–64.7%, 219.2%, and 2.3–745.7% respectively, while amylase activity was lowered. This finding was apparently not consistent with the finding that when cell wall-bound phenolic acids accumulated in the roots of resistant cultivars of date palm, they strongly reduced the cell wall-degrading enzymes of *F. o.* f. sp. *albedinis* (Mondarar and Boustani, 2001). In the case of date palm, the root extracts may have contained other compounds that lowered the enzyme levels.

It is concluded that *p*-hydroxybenzoic acid suppressed the growth of *F. o.* f. sp. *niveum*, while at the same time it stimulated the production of fusaric acid and phytopathogenic enzymes. *P*-hydroxybenzoic acid may be a means to inhibit *F. o.* f. sp. *niveum* and hence to control fusarium wilt in watermelon.

Acknowledgements

This work was financially supported by the Jiangsu Provincial Key Laboratory for Organic Solid Wastes Utilization (BM2007001). The first three authors contributed equally to this work.

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Accepted for publication: October 27, 2009